U.S. DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY'S DOCKET NUMBER
TRANMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. 371		AMITAI 1
		U S APPLICATION NO (If known,see 37 CFR 1.5) 09/807610
INTERNATIONAL APPLICATION NO. PCT/IL99/00543	INTERNATIONAL FILING DATE 14 October 1999	PRIORITY CLAIMED 14 October 1998

TITLE OF INVENTION
EXPRESSION AND SECRETION OF icIL-1 RECEPTOR ANTAGONIST TYPE II

APPLICANT(S) FOR DO/EO/US
Hagit AMITAI et al.

Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:

1. [X] This is a **FIRST** submission of items concerning a filing under 35 U.S.C. 371.
2. [ ] This is a **SECOND** or **SUBSEQUENT** submission of items concerning a filing under 35 U.S.C. 371.
3. [X] This is an express request to begin national examination procedures (35 U.S.C. 371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. 371(b) and PCT Articles 22 and 39(1).
4. [X] The US has been elected in a Demand by the expiration of 19 months from the priority date (PCT Article 31).
5. [X] A copy of the International Application as filed (35 U.S.C. 371(c)(2))
   a. [ ] is attached hereto (required only if not transmitted by the International Bureau).
   b. [X] has been communicated by the International Bureau.
   c. [ ] is not required, as the application was filed in the United States Receiving Office (RO/US).
6. [ ] An English language translation of the International Application as filed (35 U.S.C. 371(c)(2)).
7. [X] Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. 371(c)(3))
   a. [ ] are transmitted herewith (required only if not transmitted by the International Bureau).
   b. [ ] have been communicated by the International Bureau.
   c. [ ] have not been made; however, the time limit for making such amendments has NOT expired.
   d. [X] have not been made and will not be made.
8. [ ] An English language translation of the amendments to the claims under PCT Article 19 (35 U.S.C. 371(c)(3)).
9. [ ] An oath or declaration of the inventor(s) (35 U.S.C. 371(c)(4)).
10. [ ] An English language translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. 371(c)(5)).

Items 11. to 16. below concern document(s) or information included:

11. [ ] An Information Disclosure Statement under 37 CFR 1.97 and 1.98.
12. [ ] An Assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included.
13. [X] A FIRST preliminary amendment.
    [ ] A SECOND or SUBSEQUENT preliminary amendment.
14. [ ] A substitute specification.
15. [ ] A change of power of attorney and/or address letter.
16. [X] Other items or information:
    [X] Courtesy copy of the International Application as filed.
    [X] Courtesy copy of the first page of the International Publication (WO 00/22146).
    [X] Courtesy copy of the International Preliminary Examination Report. There were no annexes.
    [X] Formal drawings, 6 sheets, Figures 1A – 2C.
    [X] Courtesy Copy of the International Search Report.
    [X] Application Data Sheet

U.S. APPLICATION NO. (If known, see 37 CFR 1.5)	International Application No.	Attorney's Docket No.
09/807610	PCT/IL99/00543	AMITAI 1

17. [xx] The following fees are submitted:	CALCULATIONS PTO USE ONLY	
BASIC NATIONAL FEE (37 CFR 1.492 (a)(1) –(5):		
Neither international preliminary examination fee (37 CFR 1.482) nor international search fee (37 CFR 1.445(a)(2)) paid to USPTO and International Search Report not prepared by the EPO or JPO.....**$1000.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but International Search Report prepared by the EPO or JPO.....**$860.00**		
International preliminary examination fee (37 CFR 1.482) not paid to USPTO but international search fee (37 CFR 1.445(a)(2)) paid to USPTO.....**$710.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) but all claims did not satisfy provisions of PCT Article 33(1)-(4).....**$690.00**		
International preliminary examination fee paid to USPTO (37 CFR 1.482) and all claims satisfied provisions of PCT Article 33(1)-(4).....**$100.00**		
ENTER APPROPRIATE BASIC FEE AMOUNT =	$ 860.00	
Surcharge of **$130.00** for furnishing the oath or declaration later than [ ] 20 [X] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).	$ 130.00	

Claims as Originally Presented	Number Filed	Number Extra	Rate		
Total Claims	15 - 20		X $18.00	$	
Independent Claims	1 - 3		X $80.00	$	
Multiple Dependent Claims (if applicable)			+$270.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 990.00	
Claims After Post Filing Prel. Amend	Number Filed	Number Extra	Rate		
Total Claims	- 20		X $18.00	$	
Independent Claims	- 3		X $78.00	$	
TOTAL OF ABOVE CALCULATIONS =				$ 990.00	
Reduction of ½ for filing by small entity, if applicable. Applicant claims small entity status. See 37 CFR 1.27.				$	
SUBTOTAL =				$ 990.00	
Processing fee of **$130.00** for furnishing the English translation later than [ ] 20 [ ] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).				$	
TOTAL NATIONAL FEE =				$ 990.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). **$40.00** per property +				$	
TOTAL FEES ENCLOSED =				$ 990.00	
				Amount to be: refunded	$
				charged	$

a. [ ] A check in the amount of $_____ to cover the above fees is enclosed.
b. [X] Credit Card Payment Form (PTO-2038), authorizing payment in the amount of $ 990.00, is attached.
c. [ ] Please charge my Deposit Account No. **02-4035** in the amount of $_____ to cover the above fees. A duplicate copy of this sheet is enclosed.
d. [**XX**] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. **02-4035**. A duplicate copy of this sheet is enclosed.

NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.

SEND ALL CORRESPONDENCE TO:

BROWDY AND NEIMARK, P.L.L.C.
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WASHINGTON, D.C. 20001
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Date of this submission: **April 16, 2001**

SIGNATURE

Sheridan Neimark
NAME

20,520
REGISTRATION NUMBER

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of: Hagit AMITAI et al.	)	Art Unit:
IA No.: PCT/IL99/00543	)	Washington, D.C.
IA Filed: 14 October 1999	)	
U.S. App. No.: (Not Yet Assigned)	)	April 16, 2001
National Filing Date: (Not Yet Received)	)	
For: EXPRESSION AND...	)	Docket No.: AMITAI 1

PRELIMINARY AMENDMENT

Honorable Commissioner for Patents and Trademarks
Washington, D.C. 20231

Sir:

Contemporaneous with the filing of this case and prior to calculation of the filing fee, kindly amend as follows:

IN THE SPECIFICATION

After the title please insert the following paragraph:

REFERENCE TO RELATED APPLICATIONS

The present application is the national stage under 35 U.S.C. 371 of international application PCT/IL99/00543, filed 14 October 1999 which designated the United States, and which international application was published under PCT Article 21(2) in the English language.

In re of: Hagit AMITAI et al. (AMITAI 1)

REMARKS

The above amendment to the specification is being made to insert reference to the PCT application of which the present case is a U.S. national stage.

Favorable consideration and allowance are earnestly solicited.

Respectfully submitted,
BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant

By: ____________________
Sheridan Neimark
Registration No. 20,520

SN:wrd
Telephone No.: (202) 628-5197
Facsimile No.: (202) 737-3528

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re Application of:	)	Box PCT
AMITAI et al	)	Examiner:
Appln. No.: 09/807,610	)	Washington, D.C.
IA Appln. No. PCT/IL99/00543	)	
IA Filed: October 14, 1999	)	July 23, 2001 (MONDAY)
For: EXPRESSION AND SECRETION OF Icil-1 RECEPTOR ANTAGONIST TYPE II	)	Atty.Docket: **AMITAI=1**

RESPONSE TO NOTIFICATION TO COMPLY WITH SEQUENCE LISTING REQUIREMENTS

Honorable Commissioner for Patents
Washington, D.C. 20231

Sir:

In response to the Notification to Comply included in the Notification of Missing Requirements under 35 USC 371 dated May 14, 2001, please amend the present application as follows:

IN THE SEQUENCE LISTING

Please substitute the attached Sequence Listing, numbered as pages 1-3 for the one previously submitted.

IN THE DRAWINGS

Attached are copies of Figures 1A and 1B with revisions marked in red. Approval of these revisions is respectfully requested.

REMARKS

Applicants respectfully request the examiner's approval of revised Figures 1A and 1B. The revisions to Figures 1A and 1B are being made so that the sequences of primers P2 (Fig. 1A) and P4 (Fig. 1B) correspond to the sequences of SEQ ID NOs:8 and 10 as identified on page 4 of the specification and are complementary to the cDNA sequence as indicated by the 5'-3' direction of synthesis from primers P2 and P4.

Applicants have substituted into the present specification a new paper copy Sequence Listing section according to 37 C.F.R. §1.821(c) as new pages 1-3. Furthermore, attached hereto is a 3 1/2" disk containing the "Sequence Listing" in computer readable form in accordance with 37 C.F.R. §1.821(e).

The substitute Sequence Listing includes SEQ ID NO:11 (specification page 17, line 15), which was previously inadvertently omitted.

The following statement is provided to meet the requirements of 37 C.F.R. §1.825(a) and 1.825(b).

I hereby state, in accordance with 37 C.F.R. §1.825(a), that the amendments included in the substitute sheets of the sequence listing are believed to be supported in the application as filed and that the substitute sheets of the sequence listing are not believed to include new matter.

I hereby further state, in accordance with 37 C.F.R. §1.825(b), that the attached copy of the computer readable form

is the same as the attached substitute paper copy of the sequence listing.

Under U.S. rules, each sequence must be classified in <213> as an "Artificial Sequence", a sequence of "Unknown" origin, or a sequence originating in a particular organism, identified by its scientific name.

Neither the rules nor the MPEP clarify the nature of the relationship which must exist between a listed sequence and an organism for that organism to be identified as the origin of the sequence under <213>.

Hence, counsel may choose to identify a listed sequence as associated with a particular organism even though that sequence does not occur in nature by itself in that organism (it may be, e.g., an epitopic fragment of a naturally occurring protein, or a cDNA of a naturally occurring mRNA, or even a substitution mutant of a naturally occurring sequence). Hence, the identification of an organism in <213> should not be construed as an admission that the sequence *per se* occurs in nature in said organism.

Similarly, designation of a sequence as "artificial" should not be construed as a representation that the sequence has no association with any organism. For example, a primer or probe may be designated as "artificial" even though it is necessarily complementary to some target sequence, which may occur in nature. Or an "artificial" sequence may be a substitution mutant of a natural sequence, or a chimera of two or more natural sequences, or a cDNA (i.e., intron-free

sequence) corresponding to an intron-containing gene, or otherwise a fragment of a natural sequence.

The Examiner should be able to judge the relationship of the enumerated sequences to natural sequences by giving full consideration to the specification, the art cited therein, any further art cited in an IDS, and the results of his or her sequence search against a database containing known natural sequences.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached page is captioned "Version with markings to show changes made".

Applicants submit that the present application contains patentable subject matter and therefore urge the examiner to pass the case to issuance.

If the examiner has any questions or comments concerning the above described application, the examiner is urged to contact the undersigned at the phone number below.

Respectfully submitted,

BROWDY AND NEIMARK, P.L.L.C.
Attorneys for Applicant(s)

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SEQUENCE LISTING

<110> AMITAI, Hagit
CHITLARU, Edith

<120> EXPRESSION AND SECRETION OF icIL-1 RECEPTOR ANTAGONIST TYPE II

<130> AMITAI=1

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<141> 2001-04-16

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tatcgccatg taagcccagt atttggccaa tctcagaaag ctcctggtcc ctggagggat 180

ggagagagaa aaacaaacag ctcctggagc agggagagtg ctggcctctt gctctccggc 240

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1 5 10 15

A

Human GH Signal Peptide Genomic Sequence

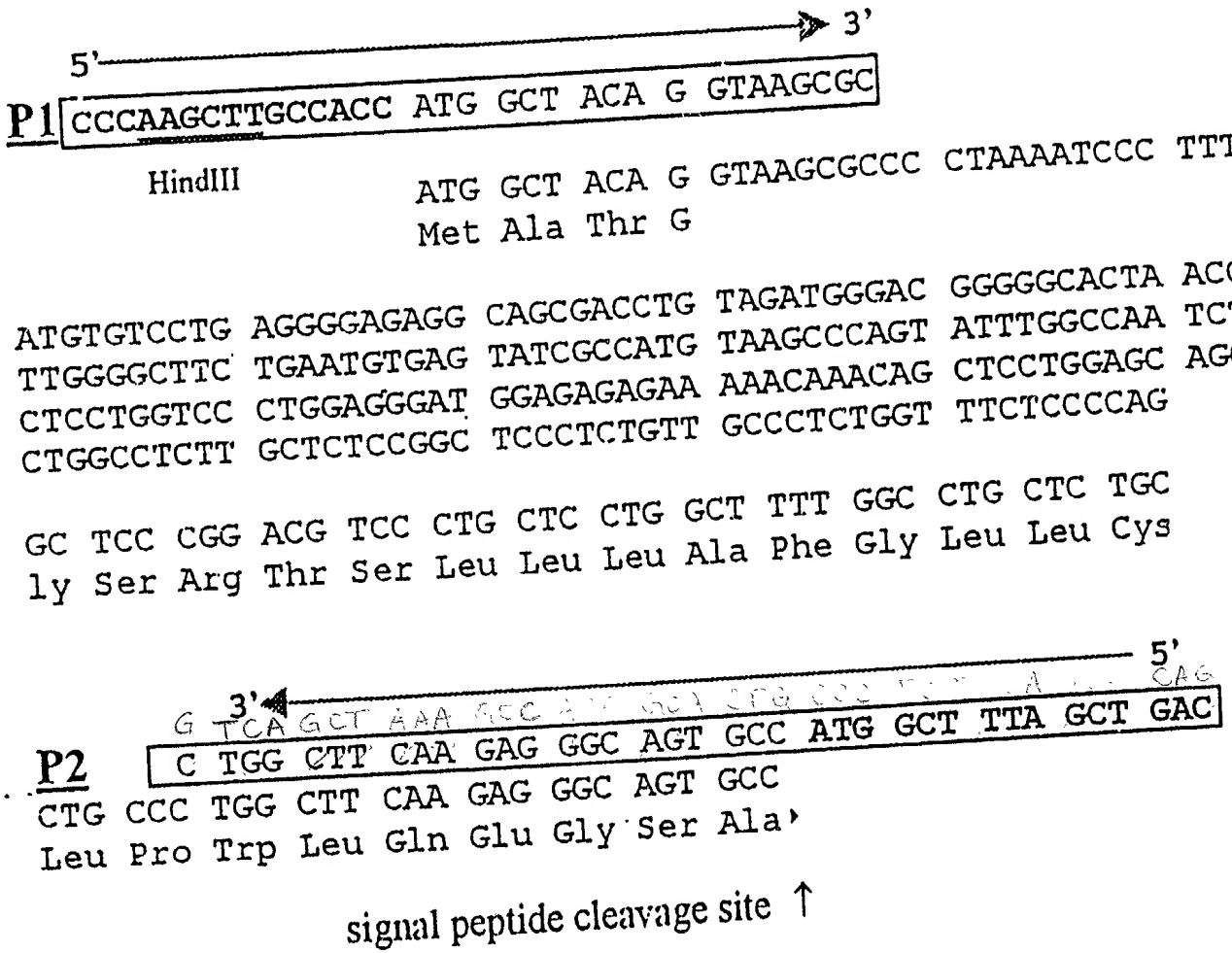
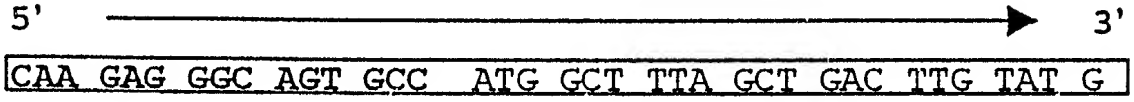


Fig. 1A

B

icIL1RaII, cDNA sequence

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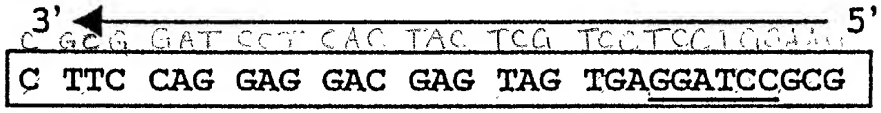

BamHI

```
GTC ACC AAA TTC TAC TTC CAG GAG GAC GAG TAG
Val Thr Lys Phe Tyr Phe Gln Glu Asp Glu ---
```

Primers are boxed, direction of synthesis is indicated by arrows, and overhangs are bold.
Enzymatic restriction sites are underlined.

Fig. 1B

EXPRESSION AND SECRETION OF icIL-1 RECEPTOR ANTAGONIST TYPE II

Field of the Invention

The present invention relates to the expression and secretion of recombinant proteins produced by DNA plasmid expression vectors in mammalian cells. More particularly this invention relates to the recombinant production of intracellular IL-1 receptor antagonist (icIL-1ra) type II by cultured COS and CHO cells, by use of DNA expression vectors containing the genomic DNA sequence of the human growth hormone (hGH) signal peptide and the cDNA of icIL-1ra type II.

Background of the Invention

IL-1 (IL-1α and IL-1β) is a pleiotropic cytokine that exerts a variety of effects on different tissues (Dinarello, 1991). IL-1 affects nearly every cell type, either alone or in synergy with other cytokines (Dinarello, 1996). Two natural pathways of negative regulation strictly control the potent inflammatory effects of IL-1, under physiological conditions. One is IL-1 receptor type II, which is a non-signaling cell-surface IL-1 binding molecule, that acts as a decoy target for IL-1 (Colotta et al, 1993; Sims et al, 1993; Colotta et al, 1994). The second is the unique, IL-1 receptor antagonist (IL-1ra) (Hannum et al, 1990; Eisenberg et al, 1990; Carter et al, 1990) polypeptide that binds both surface IL-1 receptors, and inhibits signaling from the functional IL-1 receptor.

Two forms of IL-1ra have been identified. The first was a secreted form, soluble IL-1ra (sIL-1ra), that contains a classical 25-amino acid signal peptide (Eisenberg et al, 1990; Carter et al, 1990). The second, which does not contain any signal peptide, was termed intracellular IL-1ra (icIL-1ra) (Haskill et al, 1991). icIL-1ra was in fact found to be constitutively expressed intracellularly, in

keratinocytes and in epithelial cells. icIL-1ra was shown to inhibit exogenous IL-1 dependent responses (Haskill et al, 1991).

The two IL-1ra isoforms are derived from the same gene. icIL-1ra transcript originates from an alternative start site, and splicing of an alternative first exon into an internal splice acceptor site located in the first exon of sIL-1ra (Haskill et al, 1991). These proteins are thus identical, except in their NH_2 end, in which the 21 amino acid signal peptide of sIL-1ra is substituted by three amino acids in icIL-1ra. sIL-1ra and icIL-1ra have a similar capability to inhibit IL-1 activity (Bertini et al, 1992) although expression of the two antagonists is differentially regulated (Haskill et al, 1991).

An additional isoform of icIL-1ra, termed the type II icIL-1ra, has been recently identified, cloned and functionally characterized (Muzio et al, 1995, WO 96/12022). The type II icIL-1ra contains an additional, in-frame, 63 bp sequence located three codons downstream of the translation start of icIL-1ra. This additional sequence is inserted between the first and the second exons of the intracellular form of IL-1ra. The additional exon is coded by an extra exon located 2kb downstream of the first icIL-1ra specific exon.

Human growth hormone (hGH) is a 191-amino acid protein synthesized and secreted by the somatotroph cells of the anterior pituitary. The hGH gene contains five exons and is the best characterized of the five members of the hGH gene family (DeNoto et al, 1981). *In vitro* transfection of the hGH gene into mammalian cells was found to yield high levels of secreted protein proportional to the levels of cytoplasmic hGH mRNA. Thus, secretion does not appear to be the rate-limiting step for appearance of hGH in the culture medium (Selden et al, 1986). The hGH gene includes a 26 amino acid signal peptide.

Pecceu et al (1991) discloses an attempt to use the human growth hormone signal peptide to create a hybrid gene with the mature form of interleukin-1β

(IL-1β) in order to cause mammalian cells to secrete recombinant IL-1β. Natural IL-1β is expressed initially as an intracellular 31-kDa precursor polypeptide. When proteolytic processing of the precursor occurs, secretion of a mature 17-kDa IL-1β in a soluble mature non-glycosylated form occurs. Pecceu discloses that fusion of the mature form of IL-1β to the heterologous hGH leader sequence permitted the mature IL-1β to be secreted in mature form in CHO cells, although the form which was secreted was a glycosylated form as opposed to the non-glycosylated natural form. Pecceu discloses that the glycosylated form is biologically active. However, Pecceu further states that when the biologically active part of IL-1β was preceded only by a methionine and synthesized in CHO cells, a considerable percentage of the IL-1β produced was quite unexpectedly found in the culture medium. This disclosure leaves some amount of doubt as to whether it was the hGH signal peptide which caused the expression of the IL-1β in the CHO cells or whether such expression was specific to the mechanism involved with this particular protein, the mature form of which is naturally secreted after a precursor protein is expressed intracellularly and then cleaved to form the mature protein which is secreted. Furthermore, Pecceu reports no results as to whether the non-natural glycosylated form of IL-1β creates an immunologic reaction when administered to a human or is recognized as a self protein.

Specific situations involving the recombinant production of non-secretory proteins by fusing a signal peptide of another secretory protein are disclosed in Bjorkdahl et al (1997) and Komada et al (1997).

Summary of the Invention

The present invention provides a method for the production of a recombinant intracellular protein, icIL-1ra type II, in mammalian cells. More particularly, the invention provides a process for engineering proteins to be secreted by use of a signal peptide derived from hGH in different expression vectors and to

produce the secreted proteins in different mammalian cells.

Description of the Figures

Figures 1A-1C show, in Fig. 1A, the genomic hGH signal peptide DNA sequence (SEQ ID NO:1), its amino acid sequence (SEQ ID NO:2), and the primers P1 and P2; in Fig. 1B, the beginning (SEQ ID NO:3) and the end (SEQ ID NO:5) of icIL-1ra type II cDNA, their amino acid sequences (SEQ ID NO:4 and NO:6), and primers P3 and P4 used for construction of the fusion constructs, and in Fig. 1C a schematic representation of templates and primers.

P1: hGH-sp 5' primer, containing HindIII restriction site (SEQ ID NO:7).

P2: hGH-sp 3' primer, containing 3' icIL-1ra-II sequence overhang (SEQ ID NO:8).

P3: icIL-1ra-II 5' primer, containing 5' hGH-sp sequence overhang (SEQ ID NO:9).

P4: icIL-1ra-II 3' primer, containing two stop codons and BamHI restriction site (SEQ ID NO:10).

Figures 2A-2C describe, in Fig. 2A, the construction of the pCDIC and, in Fig. 2B, the construction of pSGHIRA2 DNA vectors used for expression of the icIL-1ra type II in mammalian cells. Fig. 2C is a scheme of pDHFR.

Detailed Description of the Invention

The natural form of icIL-1ra type II is expressed intracellularly and is not secreted by the cells in which it is produced. However, in accordance with the present invention, this protein can be secreted in a mammalian recombinant production system by fusing the DNA encoding the protein to the DNA encoding the signal peptide of another human protein which is normally expressed and secreted by human

cells, and which is known to cause expression of the human protein in non-human mammalian expression systems. Preferably, the signal peptide is the 26-amino acid signal peptide of the human growth hormone gene.

Prior to the present invention, it could not have been reasonably predicted whether or not the hGH signal peptide would drive the expression of icIL-1ra-II in a mammalian cell expression system in view of the fact that icIL-1ra-II is naturally expressed only intracellularly and is not secreted from the cell. In the known prior art, as represented by Pecceu et al (1991), the hGH signal peptide was used to express and secrete the mature form of IL-1β. However, the mature form of IL-1β is naturally secreted from the cells in which it is produced, although indirectly. A precursor protein is first produced which, after intracellular processing, is secreted from the cell. However, Pecceu discloses that when a recombinant vector containing only the DNA encoding the mature form of IL-1β, without any signal protein, is used, the protein is secreted from CHO cells. Thus, it could not be predicted with a reasonable degree of certainty that a protein such as icIL-1ra-II, which is only expressed intracellularly and is not naturally secreted from the cell, could be made to be secreted in large quantities in a recombinant mammalian expression system when fused to an hGH signal peptide or to a signal peptide of another secretory protein.

The icIL-1ra-II protein produced in accordance with the present invention is glycosylated while the natural protein is non-glycosylated. Thus, the present invention further relates to the two novel glycosylated forms of icIL-1ra-II produced for the first time by means of the present invention. These are the glycosylated forms which have apparent molecular weight of approximately 27 kDa and 30 kDa as determined by Commassie blue staining of SDS-PAGE (15% acrylamide under reducing conditions). It could not be predicted with a reasonable degree of certainty whether these novel glycosylated forms of icIL-1ra-II will retain the biological activity of natural icIL-1ra-II and will not be immunogenic when

administered to humans. Experiments with these two novel glycosylated forms of icIL-1ra-II will establish that they are indeed biologically active and non-immunogenic when administered to humans.

Accordingly, the present invention is directed to a process for the recombinant expression of a protein having the amino acid sequence of natural icIL-1ra-II in a recombinant cell expression system through use of a vector which is a fusion of the signal peptide of a human secretory protein, preferably the 26 amino acid signal peptide of hGH, fused in proper reading frame with the DNA encoding icIL-1ra-II. The process comprises producing an expression vector containing DNA encoding icIL-1ra-II, either in the form of cDNA or genomic DNA, fused in proper reading frame with DNA encoding the selected signal peptide, preferably the 26 amino acid hGH signal peptide. The expression vector is then inserted into an appropriate expression host, such as CHO cells. The transformed host cells are then cultured in a manner which causes the expression vector to express its encoded protein and the expressed and secreted icIL-1ra-II protein is then collected and purified from the culture medium.

The present invention is not intended to be limited by the specific examples presented herein. While CHO cells are used as the host cells, any other eukaryotic expression system, preferably mammalian expression system, may be used such as COS cells, yeast cells, insect cells, etc. Those of ordinary skill in the art are well aware of the techniques of creating expression vectors, inserting them into expression systems and selecting clones which express the desired protein, including amplification techniques.

As would be appreciated by those skilled in the art, the types of promoters used to control transcription of the icIL-ra-II proteins may be any of those which are functional in the host cells. Examples of promoters functional in mammalian cells include the SV40 early promoter, adenovirus major late promoter,

herpes simplex (HSV) thymidine kinase promoter, rous sarcoma (RSV) LTR promoter, human cytomegalovirus (CMV)immediate early promoter, mouse mammary tumor virus (MMTV) LTR promoter. interferon-β promoter, heat shock protein 70 (hsp 70) promoter, as well as many others well known in the art. These promoters may be either constitutive or regulatable. All else being equal, constitutive promoters are preferred because an extra treatment step, such as temperature shift, addition of chemical agents or inducers, etc., is not required for expression from constitutive promoters.

The technical advance of the present invention lies in the confirmation that icIL-1ra-II can be secreted in such an expression system when using a signal peptide of a human secretory protein, preferably hGH. All of the other techniques involved are well known to those of ordinary skill in this art and can be practiced without undue experimentation using only the knowledge of the skill of the art available at the time of the present invention.

The present invention further is directed to the expression vector which contains the icIL-1ra-II DNA fused to the DNA encoding a signal protein of a human secretory protein, such as hGH, and host cells transfected with such an expression vector.

The present invention is further directed to the novel glycosylated forms of icIL-1ra-II produced in accordance with the present invention.

The invention further relates to methods for reducing the amount of IL-1 in patients having a condition involving the overexpression of IL-1, by administering one of the novel glycosylated icIL-1ra-II proteins in accordance with the present invention in a therapeutically effective amount. Appropriate therapeutic dosages for the reduction of IL-1 in patients having such a condition, can be readily empirically determined by those of ordinary skill in the art.

The glycosylated icIL-1ra-II proteins of the present invention may be

administered by any means that achieves its intended purpose. For example, administration may be by a number of different parenteral routes including, but not limited to, subcutaneous, intravenous, intradermal, intramuscular, intraperitoneal, intracerebral, intranasal, oral, transdermal, or buccal routes. Parenteral administration can be bolus injection or by gradual perfusion over time.

It is understood that the dosage administered will be dependent upon the age, sex, health, and weight of the recipient, kind of concurrent treatment, if any, frequency of treatment, and the nature of the effect desired. The total dose required for each treatment may be administered by multiple doses or in a single dose. By "effective amount", it is meant a concentration of glycosylated icIL-1ra-II protein which is capable of reducing the amount of IL-1 in patients having a condition involving elevated levels of IL-1. Such concentrations can be routinely determined by those of skill in the art. It will also be appreciated by those of skill in the art that the dosage may be dependent on the stability of the administered protein. A less stable protein may require administration in multiple doses.

The invention also relates to pharmaceutical compositions comprising the glycosylated icIL-1ra-II protein of the present invention with a pharmaceutically acceptable excipient.

Preparations for parenteral administration include sterile aqueous or non-aqueous solutions, suspensions, and emulsions, which may contain auxiliary agents or excipients which are known in the art.

Pharmaceutical compositions comprising the glycosylated icIL-1ra-II protein according to the invention include all compositions wherein the protein is contained in an amount effective to achieve its intended purpose. In addition, the pharmaceutical compositions may contain suitable pharmaceutically acceptable carriers comprising excipients and auxiliaries which facilitate processing of the active compounds into preparations which can be used pharmaceutically. Suitable

pharmaceutically acceptable vehicles are well known in the art and are described for example in Gennaro, Alfonso, Ed., Remington's Pharmaceutical Sciences, 18th Edition, Mack Publishing Co., Easton, PA (1990), a standard reference text in this field. Pharmaceutically acceptable vehicles can be routinely selected in accordance with the mode of administration and the solubility and stability of the protein. For example, formulations for intravenous administration may include sterile aqueous solutions which may also contain buffers, diluents and other suitable additives.

While any excipients known for the administration of therapeutic proteins can be used in accordance with the present invention, excipients used for intravenous administration are preferred.

Suitable formulations for parenteral administration include aqueous solutions of the active compounds in water-soluble form, for example, water-soluble salts. In addition, suspension of the active compound as appropriate oily injection suspensions may be administered. Suitable lipophilic solvents or vehicles include fatty oils, for example, sesame oil, or synthetic fatty acid esters, for example, ethyl oleate or triglycerides. Aqueous injection suspensions that may contain substances which increase the viscosity of the suspension include, for example, sodium carboxymethyl cellulose, sorbitol, and/or dextran. Optionally, the suspension may also contain stabilizers.

The vectors in accordance with the present invention can also be used in gene therapy to cause appropriate human cells to express icIL-1ra-II *in vivo* in order to direct the IL-1 antagonizing effect of this protein directly at the desired site. The novelty of this process lies in the particular vector and the knowledge of the activity of the glycosylated protein produced thereby and not in the specific methods of gene therapy, including the methods of introducing an expressible vector directly into the cells of interest. These are within the skill of those of ordinary skill in this art at the time the present invention was made.

Non-limiting specific examples of the present invention follow.

Example 1: Generation of hGH-sp-icIL-ra-II Fragment

The human growth hormone hGH signal peptide sequence was amplified by PCR using the pXGH5 vector, which encodes the full-length human growth hormone genomic sequence, as template (DeNoto et al, 1981). pXGH5 was used as a template for PCR using primers P1 (containing a HindIII restriction site and a Kozak sequence) and P2 (which has a 3' icIL-1ra-II sequence overhang, Fig. 1A). The icIL-1ra-II cDNA was amplified by PCR with primers P3 (which has a 5' hGH signal peptide sequence overhang) and P4 (which has two stop codons and a BamHI restriction site, Fig. 1B). These two PCR fragments were annealed together by their homologous regions and were further amplified by PCR using primers P1 and P4, to generate the hGH-icIL-1ra-II fragment (Fig. 1C).

Example 2: Construction of pCDIC

hGH-sp-icIL-1ra-II fragment was digested with HindIII and BamHI and cloned into the HindIII-BamHI sites of pCDNA3.1 (+) (Invitrogen, San Diego, Fig. 2A) downstream of the CMV promoter. The resulting vector (pCDIC, Fig. 2A) was mapped by restriction analysis, and used to transfect COS cells.

Example 3: Construction of pSGHIRA2

hGH-sp-icIL-1ra-II fragment was digested with HindIII and BamHI, and cloned into HindIII-BclI sites of pSVE3 (Fig. 2B; Hartman et al, 1982), downstream of the early SV-40 promoter. The resulting vector pSGHIRA2 (Fig. 2B) was used to transfect CHO DUKX (ATCC, CRL 9010) cells in co-transfection with the mouse DHFR containing vector pDHFR (Fig. 2C) as detailed below. The constructs were analyzed by restriction mapping and sequenced.

Example 4: Expression Vector Carrying the Mouse DHFR Gene

Plasmid pDHFR (Fig. 2C) is composed of the complete pBR322 sequence. the SV40 early gene promoter, the 70 bp splicing region of the mouse γ2a gene fused to the mouse DHFR cDNA, followed by the SV40 early gene polyadenylation signal.

Example 5: Transient Expression in COS Cells

pCDIC DNA was used for transfecting COS cells by means of the DEAE dextran method. Cells were seeded at approximately $3x10^6/80$ cm^2 flask and allowed to grow overnight. The next day all medium was aspirated from the flasks and 5 ml of transfection medium was added to the cells. Transfection medium contains 400 µg/ml DEAE dextran 100 µM Chloroquine, 2 µg/ml DNA, and 10% NuSerum in RPMI medium. After incubation for 3-4 hrs at 37°C the transfection medium was removed by aspiration and replaced with 5 ml of 10% DMSO in PBS for 2 minutes at room temperature. This solution was then aspirated and culture medium containing 10% FBS in RPMI added to the flasks. The cultures were incubated at 37°C for 24 hours, then the culture medium was changed to medium containing 2% serum. 24 hours later incubation temperature was reduced to 32°C, and culture supernatant samples were analyzed for the presence of the icIL-1ra type II in the culture supernatant by ELISA (see Example 7). 6-7 $µg/1x10^6$ cells/run icIL-1ra type II were secreted from the transfected COS cells. Highest production levels were at days 4 to 8 following transfection. These results indicate that fusion of the hGH signal peptide to the intracellular form of IL-1-1ra type II, enables its secretion into the culture medium of the transfected cells.

Example 6: Stable Expression in CHO Cells

CHO cells were cotransfected with pSGHIRA2 vector carrying the genes for icIL-1ra type II and with pDHFR carrying the gene for mouse DHFR (described in Fig. 2C), by means of the Lipofectamine transfection method.

Cells were seeded $1x10^6$/10 cm plate in F12 medium containing 10% FCS, and allowed to grow overnight. The cells were washed in F12 medium, and 8 ml of the DNA-Lipofectamine mixture was added to the plates, which were then incubated for 4-5 hours at 37°C. At the end of the incubation period, 8 ml of F12 medium containing 20% FCS were added, and the plates were incubated for 24 hours at 37°C. The culture medium was then changed to fresh F12 medium containing 10% FCS. 72 hours following transfection cell cultures were seeded, either by limiting dilution, or by subculture at a 1:20 dilution, into selective medium depleted of Thymidine and Hypoxantine, containing 10% dialyzed FCS, and allowed to grow until single colonies could be picked and analyzed. Expression of icIL-1ra-II by eight stably integrated CHO clones is summarized in Table 1. The process lends itself to scale up by methods known in the art.

Table 1

Specific Productivity of icIL-1ra-II by CHO Clones

Clone Number	Specific Productivity ng/10^6 cells/day	
	Before MTX Amplification	After MTX Amplification (up to 400 nM MTX)
1-33	118	273
2-56	91	220
1-64	122	186
1-84	194	245
2-2	92	524
2-66	124	283
2-73	82	218
2-88	120	442

The MTX amplification was performed as follows:

Cells that grew in the absence of MTX were seeded to six T-flasks, in the presence of different MTX concentrations (e.g., 0, 2nM, 5nM, 10nM, 20nM, 50nM). About 10 days later, the cultures were observed microscopically and the cells were counted, in order to determine survival. The MTX concentration that allowed the survival of approximately 10% of the culture was selected for further propagation. The second round of amplification was performed in a similar manner, however the MTX concentrations were higher, starting from the MTX concentration that was selected at the first round. The cultures were again scored for survival, relative to the control MTX concentration of this round. The clones presented in Table 1 were amplified to the MTX concentrations shown in Table 2.

Table 2

Clone	MTX First Round	MTX Second Round
1-33	20nM	300nM
1-64	20nM	400nM
1-84	20nM	100nM
2-2	50nM	200nM
2-56	50nM	100nM
2-66	20nM	100nM
2-73	20nM	100nM
2-88	20nM	100nM

Example 7: ELISA Test

Microtiter plates (Nunc) were coated with mouse anti IL-1ra antibody (purified ascitis IgG, MCA 1467, clone 1384, Serotec Ltd, Oxford, UK) 5 μg/ml in PBS (100 μl/well), for 3 hrs at 37°C, and stored 40°C. The plates were washed with PBS containing Tween 20 (0.05%, referred to herein as washing buffer) and blocked with the same solution containing 1% bovine serum albumin (BSA, referred to herein as blocking solution) for 1 hour at 37°C. Plates were then washed in washing buffer. The samples to be analyzed were diluted in the blocking solution, and added to the wells (100 μl/well) for 90 minutes at 37°C. The plates were then washed 6 times in washing buffer, followed by addition of biotinylated anti human IL-1ra antibody (100 μl/well of a 1:10,000 dilution, MCA 1466B, clone 1390m, Serotec, Oxford, UK). Plates were incubated for 1 hr at 37°C and washed with washing buffer. A horseradish peroxidase (HRP) streptavidin conjugate (1 mg/ml Sigma, Rehovot, Israel, 100 μl/well diluted 1:10,000 in blocking buffer) was then added to the plates, and incubated for 1.5 hours at 37°C. The plates were then washed in buffer and the substrate solution (*o*-phenylenediamine dihydrochloride, OPD, Sigma Rehovot, Israel, 100 μl/well) was added for 10 min. at 22°. The reaction was stopped by addition of 100 μl/well of 4N HCl. The plates were then read in an automated Elisa reader. A standard preparation of IL-1ra (Serotec, Oxford, UK, PHP080, 2-128 ng/ml) was used as reference for the IL-1ra concentration.

Example 8: Affinity Chromatography of icIL-1ra Type II with Monoclonal Antibodies

Affinity chromatography of icIL-1ra type II was performed by binding anti-human IL-1ra antibodies (purified ascitis IgG, MCA 1467, clone 1384, Serotec Ltd, Oxford, UK) to CNBr activated Sepharose 4B (5 mg/ml resin, Pharmacia,

Uppsala, Sweden). Culture supernatant from CHO cells, of clone 2-88 of the above-mentioned Example 6 was diafiltrated over a 100K membrane and then concentrated over a 10K membrane. Concentrated proteins were dialyzed against 0.1 M $NaHCO_3$, 150 mM NaCl pH 8.2. This procedure enriched the product concentration, reduced the volume of sample (100 fold) and removes major impurities. The yield of this step is about 85%. 30 ml of concentrated proteins were loaded on a 3.2 ml column, that had been equilibrated with 0.1 M sodium carbonate, 150 mM sodium chloride, pH 8.3, at a flow rate of 2 ml/min. icIL-1ra was eluted in 150 mM citric acid, 300 mM NaCl pH 2.7. Eluted fractions were immediately neutralized with 1M Tris pH 9.3. The fraction eluted resolved into two bands of apparent molecular weight of approximately 27 kDa and 30 kDa respectively, as determined by Commassie blue staining of SDS-PAGE (15% acrylamide under reducing conditions). The different molecular weights are presumably due to variations in glycosylation.

Example 9: Western Blot

The affinity chromatography column eluate of above-mentioned Example 8 was concentrated by filtration on a 3K membrane (Miniset, Pall Filtron, Northborough, MA). Fractions were resolved on a 15% acrylamide SDS-PAGE gel under reducing conditions (Readygel BioRad, Hercules, CA) and electroblotted onto a nitrocellulose membrane (BRL, Life Technologies, MD). The blot was incubated in PBS containing 10% low fat milk, 0.1% Tween 20, overnight. The blot was then incubated with mouse anti human IL-1ra antibodies (purified ascitis IgG 1:5,000, MCA 1467, Serotec Ltd, Oxford, UK) for 2 hours at RT, then washed three times for 15 minutes in PBS containing 0.1% Tween 20, and further incubated with goat anti-mouse horseradish peroxidase-alkaline phosphatase (1:10,000 Sigma, Israel) for 1 hour at RT. The blot was then washed 3 times in PBS containing 0.1% Tween 20, followed by detection with enhanced luminescence (Amersham). Two protein bands

of approximately 27 kDa and 30 kDa, respectively, corresponding to icIL-1ra, were identified.

Example 10: Protein Sequence Analysis

The purified fraction from the immunoaffinity chromatography column of Example 8 was electroblotted in parallel, both on a PVDF membrane (Millipore, Bedford, MA), and on a nitrocellulose membrane for Western blotting analysis as described in the above-mentioned Example 9. The two bands stained by Coommassie blue were both recognized as IL-1ra by Western blot analysis. The purified fraction eluted from the affinity chromatography column, as well as the two bands excised from the Coomassie blue stained PVDF membrane, were subjected to protein sequence analysis by Edman degradation in the Procise™, 491HT microsequencer (Applied Biosystems, USA). Sequencing of the N-terminal amino acids, indicated that the purified fraction separated from the culture supernatant contained two forms of icIL-1ra. The amino acid sequence obtained, ALADLYEEGGGGGGE (SEQ ID NO:11), demonstrated that the secreted protein represented the mature icIL-1ra type II protein beginning at amino acid position +2 from the deduced start of translation of the gene (GENBANK, ID# X84348). An additional icIL-1ra form, beginning at amino acid +1 from the deduced start of translation of the icIL-1ra type II, was found as well.

Having now fully described this invention, it will be appreciated by those skilled in the art that the same can be performed within a wide range of equivalent parameters, concentrations, and conditions without departing from the spirit and scope of the invention and without undue experimentation.

While this invention has been described in connection with specific embodiments thereof, it will be understood that it is capable of further modifications. This application is intended to cover any variations, uses, or adaptations of the

inventions following, in general, the principles of the invention and including such departures from the present disclosure as come within known or customary practice within the art to which the invention pertains and as may be applied to the essential features hereinbefore set forth as follows in the scope of the appended claims.

All references cited herein, including journal articles or abstracts, published or unpublished U.S. or foreign patent applications, issued U.S. or foreign patents, or any other references, are entirely incorporated by reference herein, including all data, tables, figures, and text presented in the cited references. Additionally, the entire contents of the references cited within the references cited herein are also entirely incorporated by reference.

Reference to known method steps, conventional method steps, known methods or conventional methods is not in any way an admission that any aspect, description or embodiment of the present invention is disclosed, taught or suggested in the relevant art.

The foregoing description of the specific embodiments will so fully reveal the general nature of the invention that others can, by applying knowledge within the skill of the art (including the contents of the references cited herein), readily modify and/or adapt for various applications such specific embodiments, without undue experimentation, without departing from the general concept of the present invention. Therefore, such adaptations and modifications are intended to be within the meaning and range of equivalents of the disclosed embodiments, based on the teaching and guidance presented herein. It is to be understood that the phraseology or terminology herein is for the purpose of description and not of limitation, such that the terminology or phraseology of the present specification is to be interpreted by the skilled artisan in light of the teachings and guidance presented herein, in combination with the knowledge of one of ordinary skill in the art.

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CLAIMS

1. An expression vector, comprising a DNA segment encoding a signal peptide of a protein which is normally expressed and secreted by human cells, joined to a DNA segment encoding intracellular IL-1 receptor antagonist type II (icIL-1ra-II) and operably linked to a promoter sequence, wherein said icIL-1ra-II is expressed from said promoter sequence and translated with said signal peptide fused in frame to icIL-1ra-II.

2. An expression vector in accordance with claim 1, wherein said signal peptide is human growth hormone signal peptide.

3. A host cell transformed with the expression vector of claim 1.

4. A host cell transformed with the expression vector of claim 2.

5. A host cell in accordance with claim 3, wherein said cell is an endogenous cell of a human host.

6. A host cell in accordance with claim 4, wherein said cell is an endogenous cell of a human host.

7. A method for producing a recombinant icIL-1ra-II comprising the steps of:

culturing a host cell according to claim 3 to express and produce a recombinant glycosylated icIL-1ra-II;

recovering the produced recombinant glycosylated icIL-1ra-II.

8. A method for producing a recombinant icIL-1ra-II comprising the steps of:

culturing a host cell according to claim 4 to express and produce a recombinant glycosylated icIL-1ra-II;

recovering the produced recombinant glycosylated icIL-1ra-II.

9. A glycosylated icIL-1ra-II produced by a method according to claim 7.

10. The glycosylated icIL-1ra-II according to claim 9 having an apparent molecular weight of about 27 kDa on SDS-PAGE under reducing conditions with 15% acrylamide.

11. The glycosylated icIL-1ra-II according to claim 9 having an apparent molecular weight of about 30 kDa on SDS-PAGE under reducing conditions with 15% acrylamide.

12. A pharmaceutical composition, comprising the glycosylated icIL-1ra-II according to claim 9 in a therapeutically effective amount and a pharmaceutically acceptable excipient.

13. A method for reducing the amount of IL-1 in a patient having a condition associated with overexpression of IL-1, comprising administering the pharmaceutical composition according to claim 12 to a patient in need thereof.

14. A method for reducing the amount of IL-1 at a desired site in a human patient, comprising introducing a vector in accordance with claim 3 into appropriate endogenous human cells at the desired site to produce transformed cells which will express icIL-1ra-II at the desired site.

15. A method for reducing the amount of IL-1 at a desired site in a human patient, comprising introducing a vector in accordance with claim 4 into appropriate endogenous human cells at the desired site to produce transformed cells which will express icIL-1ra-II at the desired site.

ABSTRACT OF THE DISCLOSURE

Novel glycosylated intracellular IL-1 receptor antagonist type II (icIL-1ra-II) is expressed and secreted in mammalian cells transformed with an expression vector where icIL-1ra-II is secreted by expressing icIL-1ra-II fused to the human growth hormone signal peptide. Also disclosed are a pharmaceutical composition containing glycosylated icIL-1ra-II as an active ingredient and a method for reducing IL-1 levels in patients having a condition involving overexpressed IL-1.

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A

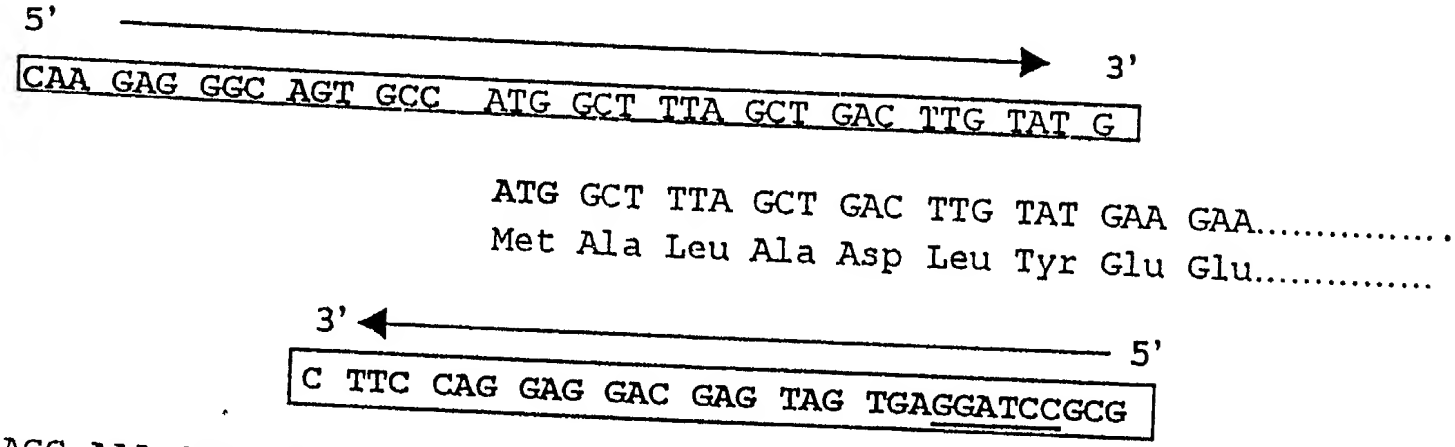
Human GH Signal Peptide Genomic Sequence

```
       5'------------------------------------> 3'
P1  CCCAAGCTTGCCACC ATG GCT ACA G GTAAGCGC
      HindIII
                    ATG GCT ACA G GTAAGCGCCC CTAAAATCCC TTTGGGCACA
                    Met Ala Thr G

ATGTGTCCTG AGGGGAGAGG CAGCGACCTG TAGATGGGAC GGGGGCACTA ACCCTCAGGT
TTGGGGCTTC TGAATGTGAG TATCGCCATG TAAGCCCAGT ATTTGGCCAA TCTCAGAAAG
CTCCTGGTCC CTGGAGGGAT GGAGAGAGAA AAACAAACAG CTCCTGGAGC AGGGAGAGTG
CTGGCCTCTT GCTCTCCGGC TCCCTCTGTT GCCCTCTGGT TTCTCCCCAG

GC TCC CGG ACG TCC CTG CTC CTG GCT TTT GGC CTG CTC TGC
ly Ser Arg Thr Ser Leu Leu Leu Ala Phe Gly Leu Leu Cys

            3'<---------------------------------------------- 5'
P2      C TGG CTT CAA GAG GGC AGT GCC ATG GCT TTA GCT GAC
CTG CCC TGG CTT CAA GAG GGC AGT GCC
Leu Pro Trp Leu Gln Glu Gly Ser Ala

                 signal peptide cleavage site ↑
```

Fig. 1A

B

icIL1RaII, cDNA sequence



Primers are boxed, direction of synthesis is indicated by arrows, and overhangs are bold. Enzymatic restriction sites are underlined.

Fig. 1B

C

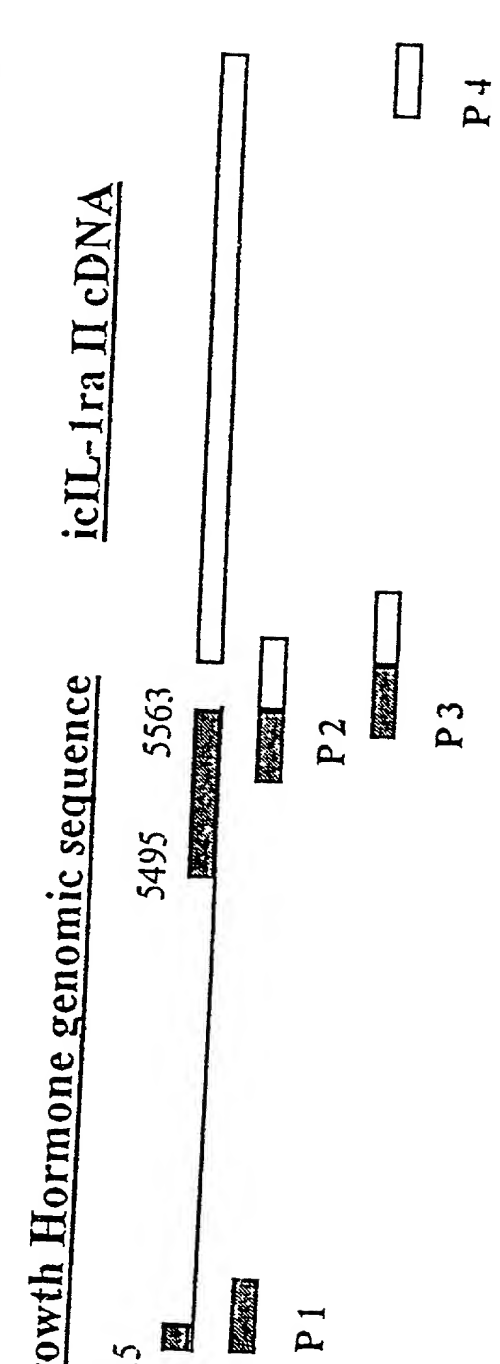
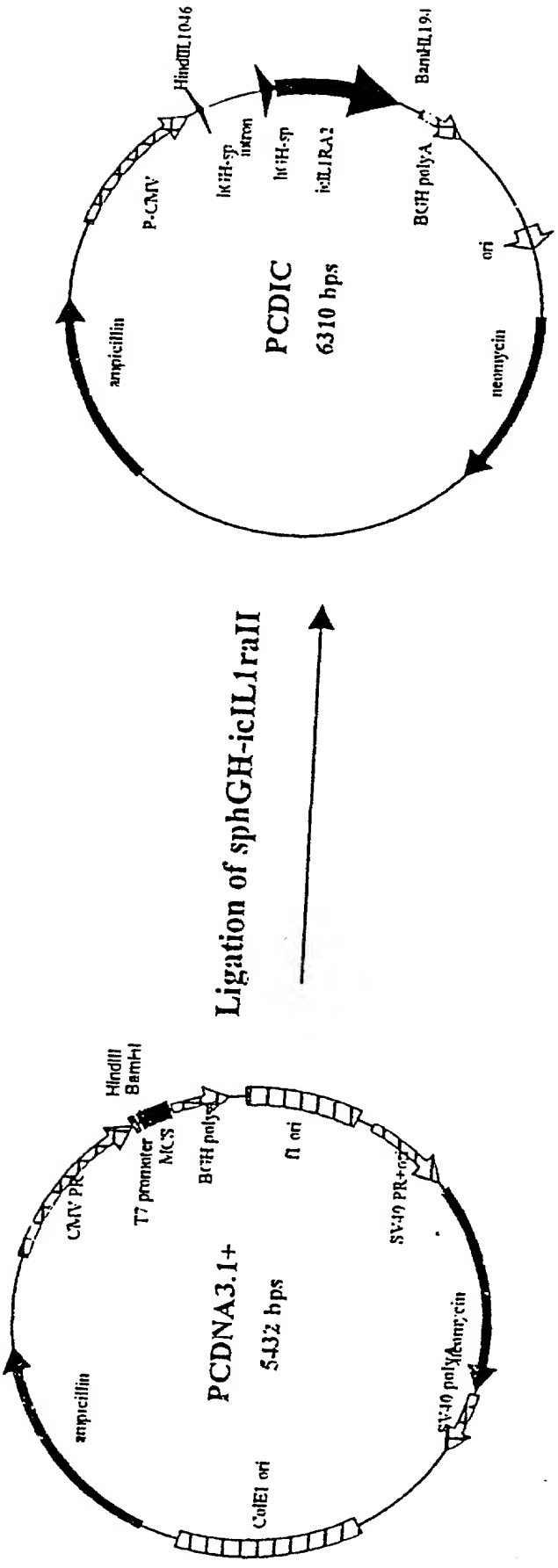

Schematic representation of templates and primers
Growth Hormone genomic sequence
icIL-1ra II cDNA
5225
5495
5563
P1
P2
P3
P4

Fig. 1C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ori
ampicillin
neomycin
Ligation of sphGH-icIL1raII
PCDNA3.1+
5432 bps
CMV PR
T7 promoter
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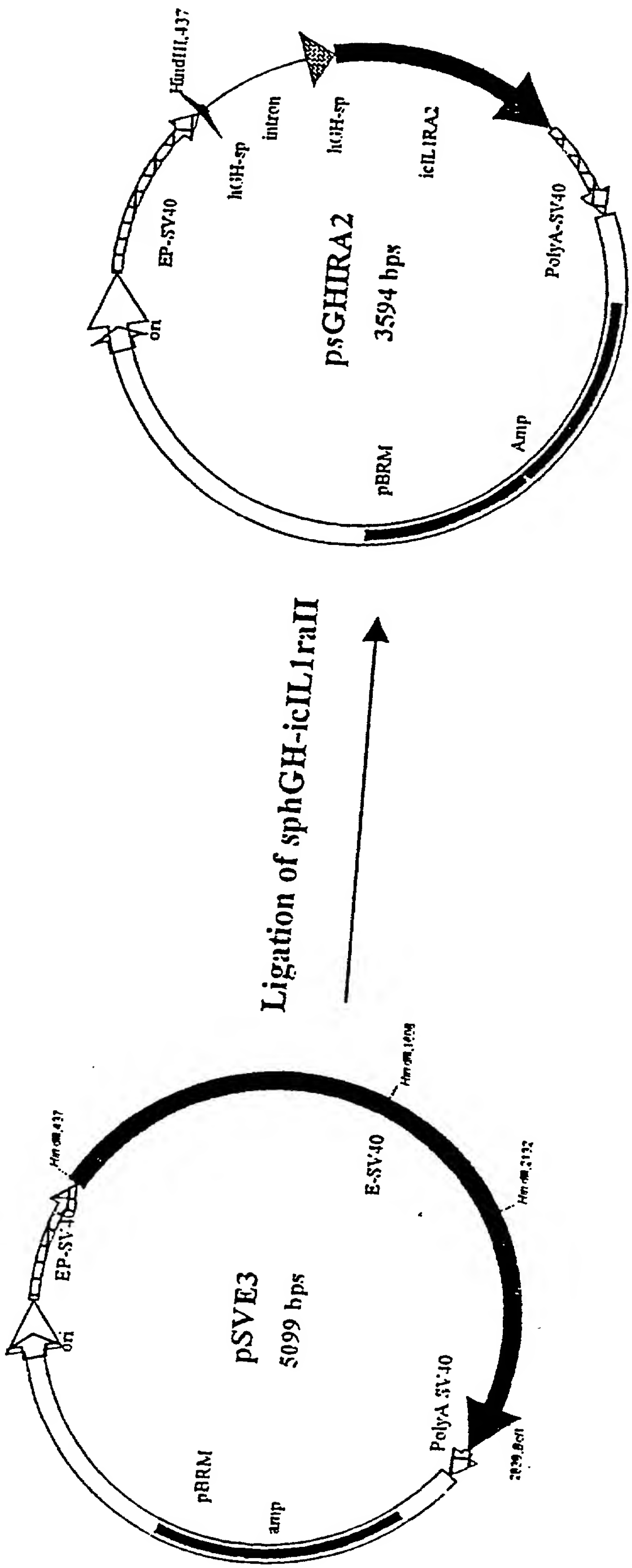
neomycin
ampicillin
ColE1 ori
HindIII
BamHI

Fig. 2A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hGH-sp
intron
hGH-sp
icIL1RA2
EP-SV40
psGHIRA2
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PolyA-SV40
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Amp
Ligation of sphGH-icIL1raII
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amp

Fig. 2B

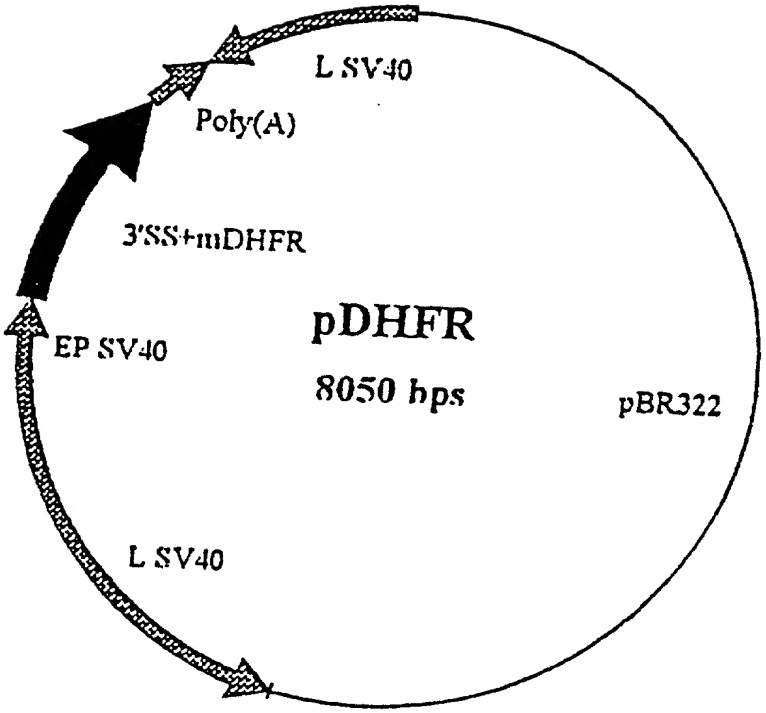

Fig. 2C

Page 1 of 2 Pages [ ] Original [ ] Substitute [ ] Supplemental Atty. Docket:

Combined Declaration for Patent Application and Power of Attorney

As a below-named inventor, I hereby declare that:

My residence, post office address and citizenship are as stated below next to my name; and that I believe I am the original, first and sole inventor (if only one name is listed below) or an original, first and joint inventor (if plural names are listed below) of the subject matter which is claimed and for which a patent is sought on the invention entitled

Expression and Secretion of icIL-1 Receptor Antagonist Type II

the specification of which (check one)

[ ] is attached hereto;
[ ] was filed in the United States under 35 U.S.C. §111 on April 16, 2001, as U.S. Appln. No. 09/807,610 or
[ ] was/will be filed in the U.S. under 35 U.S.C. §371 by entry into the U.S. national stage of an international (PCT) application, PCT/_____; filed _____, entry requested on _____*; national stage application received U.S. Appln. No. _____*; §371/§102(e) date _____* (* if known)

and was amended on _____ (if applicable).
(include dates of amendments under PCT Art. 19 and 34 if PCT)

I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above; and I acknowledge the duty to disclose to the Patent and Trademark Office (PTO) all information known by me to be material to patentability as defined in 37 C.F.R. §1.56.

I hereby claim foreign priority benefits under 35 U.S.C. §§ 119 (a)-(d) and 365 (b) of any prior foreign application(s) for patent or inventor's certificate, or §365(a) of any prior PCT application(s) designating a country other than the U.S., listed below with the "Yes" box checked, and have also identified below, by checking the "No" box, any foreign application for patent or inventor's certificate or PCT international application having a filing date before that of the application on which priority is claimed:

(Number)	(Country)	(Day Month Year Filed)		
126562	Israel	14 Oct. 1998	[X] YES	[ ] NO
			[ ] YES	[ ] NO

I hereby claim the benefit under 35 U.S.C. §119(e) of any United States provisional applications listed below:

(Application No.)	(Day Month Year Filed)

I hereby claim the benefit under 35 U.S.C. §120 of any prior U.S. non-provisional application(s) or under §365(c) of any prior PCT international application(s) designating the U.S., listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such U.S. or PCT international application in the manner provided by the first paragraph of 35 U.S.C. §112, I acknowledge the duty to disclose to the PTO all information which is material to patentability as defined in 37 C.F.R. §1.56 which became available between the filing date of the prior application and the national or PCT international filing date of this application:

(Application No.)	(Day Month Year Filed)	(Status: patented, pending, abandoned)
PCT/IL99/00543	10 October 1999	

As a named inventor, I hereby appoint the following registered practitioners to prosecute this application and to transact all business in the Patent and Trademark Office connected therewith:

All of the practitioners associated with Customer Number 001444

Direct all correspondence to the address associated with **Customer Number 001444**, which is presently:

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The undersigned hereby authorizes the U.S. Attorneys or Agents appointed herein to accept and follow instructions from Interpharm Laboratories Ltd. as to any action to be taken in the U.S. Patent and Trademark Office regarding this application without direct communication between the U.S. Attorneys or Agents and the undersigned. In the event of a change of the persons from whom instructions may be taken, the U.S. Attorneys or Agents appointed herein will be so notified by the undersigned.

Atty. Docket:

Title: Expression and Secretion of icIL-1 Receptor Antagonist Type II

U.S. Application filed _____, Serial No. _____

PCT Application filed _____, Serial No. _____

I hereby further declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under 18 U.S.C. §1001 and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

1-00

FULL NAME OF FIRST INVENTOR	INVENTOR'S SIGNATURE	DATE
Hagit Amitai		16/07/01
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2-00

FULL NAME OF SECOND JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
Edith Chitlaru	Edith Chitlaru	16/07/01
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FULL NAME OF THIRD JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
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FULL NAME OF FOURTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		

FULL NAME OF FIFTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		

FULL NAME OF SIXTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		

FULL NAME OF SEVENTH JOINT INVENTOR	INVENTOR'S SIGNATURE	DATE
RESIDENCE	CITIZENSHIP	
POST OFFICE ADDRESS		

ALL INVENTORS MUST REVIEW APPLICATION AND DECLARATION BEFORE SIGNING. ALL ALTERATIONS MUST BE INITIALED AND DATED BY ALL INVENTORS PRIOR TO EXECUTION. NO ALTERATIONS CAN BE MADE AFTER THE DECLARATION IS SIGNED. ALL PAGES OF DECLARATION MUST BE SEEN BY ALL INVENTORS.